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Forskolin and phorbol ester have opposite effects on the expression of mucin-associated sialyl-Lewis[a] in pancreatic cancer cells

Y. Yamashita [a], J.J.L. Ho [b,*], E.R. Farrelly [b], K. Hirakawa [a], M. Sowa [a], Y.S. Kim [b]

[a] *First Department of Surgery, Osaka City University Medical School, Osaka, Japan*
[b] *Gastrointestinal Research Laboratory (151M2), Veterans Affairs Medical Center and Department of Medicine, University of California, San Francisco, CA, 84121, USA*

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Abstract

The carbohydrate antigen sialyl-Lewis[a] is important to pancreatic tumour biology because the circulating antigen is used in serological tests for malignancy and because cell surface antigen is involved in tumour cell binding to the endothelial adhesion molecule, E-selectin, in extravasation. In this study, we examined the effects of the adenylyl cyclase activator, forskolin, and the diacylglycerol analogue, phorbol 12-myristate 13-acetate (PMA), on the expression and release of sialyl-Lewis[a] in human pancreatic cancer cells. Increases in the release of sialyl-Lewis[a] from SW1990 cells produced by forskolin and PMA were associated with increases in the activities of protein kinases A and C, respectively, and could be blocked by inhibitors specific for these enzymes. Immunoprecipitation experiments showed that sialyl-Lewis[a] was associated with MUC1 mucin. Forskolin also increased the cellular content of antigen and MUC1 mRNA. Actinomycin D and a protein kinase A inhibitor, H8, blocked these effects. In contrast, PMA reduced cellular antigen and MUC1 mRNA levels, although it produced a temporary increase in release of the antigen. The effects of PMA were blocked by the protein kinase C inhibitor, H7. PMA also reduced cell binding to the adhesion molecule E-selectin. In summary, PKA and PKC alter cell MUC1-associated sialyl-Lewis[a] in opposite directions. These changes may have clinical utility in the diagnosis of pancreatic cancer and the prevention of metastases.

Keywords: Sialyl-Lewis[a]; cAMP; Phorbol ester; Mucin; Pancreas; E-selectin

1. Introduction

One of the reasons that mucin-associated sialyl-Lewis[a] is important to pancreatic tumour biology is that it is the basis of several immunodiagnostic tests for malignancy such as CA19-9 [1] and one developed in our laboratory, SPan-1 [2,3]. Although mucins have shown promise in tumour diagnosis, their use is limited because not all tumours, or all cells within a tumour, express the antigens. Moreover, circulating levels of mucin antigens are low during early stages of tumorigenesis. Information on how the expression and release of mucins can be increased could be used to improve the sensitivities of mucin-based diagnostic tests. However, mucins also have actions that promote tumorigenesis [4]. For example, binding of pancreatic tumour cells to the endothelial cell adhesion molecule E-selectin via their surface sialyl-Lewis[a] [5,6] is thought to be one of the early steps in their extravasation. Thus, knowledge of mechanisms that will alter cellular levels of mucin-associated sialyl-Lewis[a] may have clinical applicability in the diagnosis and control of pancreatic malignancies.

Little is known about the intracellular signal transduction pathways that regulate the synthesis and release of mucin-associated sialyl-Lewis[a] in pancreatic cancer cells. Two of the pathways involve cyclic AMP and hydrolysis products of inositol phospholipids. In this report, we investigated whether forskolin (an activator of adenylyl cyclase) and phorbol 12-myristate 13-acetate (PMA, an analogue of 1,2-diacylglycerol) could increase the release of mucin-associated sialyl-Lewis[a] from pancreatic cancer cells, and also examined their effects on the cellular content of antigen.

* Corresponding author. Tel.: +1-415-750-2095; fax: +1-415-750-6972.
E-mail address: jjl@itsa.ucsf.edu (J.J.L. Ho).

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2. Materials and methods

2.1. Cell culture

SW1990 human pancreatic cancer cells were cultured in Dulbecco's modified Eagle's medium, DMEM (GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) [3]. Conditioned media (serum-free) and cells previously sonicated for 20 sec were centrifuged at 100 000*g* for 1 h. Media were concentrated with P10 Centricon concentrators (Amicon, Beverly, MA, USA).

Chemicals used in this study were obtained from Sigma Chem. Co. (St Louis, MO, USA) unless otherwise specified. H7 was obtained from Calbiochem (San Diego, CA, USA). Stock solutions of actinomycin (Act D), forskolin, H7, H8 and PMA were made in dimethylsulphoxide (DMSO). The viability of cells was tested with the vital stain 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [7]. Cells were treated in serum-free media. Inhibitors were added to cells 30 min prior to the activators.

2.2. Protein kinase assays

PKA (protein kinase A) and PKC (protein kinase C) assays are based on the transfer of radioactive phosphate from ATP to a specific peptide substrate and binding of the phosphorylated peptide to phosphocellulose paper. PKA: lysates were produced by suspending cells in 10 mM NaH_2PO_4, 10 mM EDTA, 0.5 mM 3-isobutyl-1-methylxanthine, 50 mM NaCl and 0.2% Triton X-100 (pH 6.8) and sonicating for 20 sec. PKA activity was assayed with LRRASLG (Kemptide) as substrate (a kit from Upstate Biotech., Lake Placid, NY, USA). In some assays a PKA inhibitor (0.5 μM of TYADFIASGRTGRRNAI, Upstate Biotech.) was included [8]. PKC: cells were suspended in 20 mM 3-[N-morpholino] propane sulphonic acid (MOPS), 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 0.25 M sucrose and 0.5% Triton X-100 (pH 7.4) and sonicated for 20 s. Assays were performed on the same day using QKRPSQRSKYL as the substrate (a kit from Upstate Biotech.) In some assays a PKC inhibitor (0.4 μM of RFARKGALRQKNV, Upstate Biotech.) was included [9]. Adenosine 5′ triphosphate, tetra (triethylammonium) salt [γ-^{32}P] of (sp. act., 6000 Ci/mmole) was from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA).

2.3. Antibodies

The 19-9 hybridoma was from the American Type Culture Collection (Rockville, MD, USA). 139H2 monoclonal antibody (MAb) was a gift from J. Hilkens (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [10]. 139H2 was purified on protein A columns (Econo-Pac Protein A kit, Bio-Rad, Hercules, CA, USA). CSLEX-1 MAb was from Signet Lab., Inc., Dedham, MA, USA. SNH3 MAb [11] ascites was a gift from S.-I. Hakomori (The Biomembrane Institute, U. of Washington, Seattle, WA, USA). The SPan-1 hybridoma was developed in our laboratory [3]. The MAb against E-selectin was from Genzyme (Cambridge, MA, USA).

2.4. Enzyme-linked immunoassays

Cells grown in microtitre plates were fixed with 10% formalin for 10 min. Comparisons of levels of soluble antigens were on the basis of the same number of cells. Assays were performed as previously described [12]. In double determinant assays, an optimal dilution of purified 139H2 was first adsorbed to the wells of microtitre plates (E.I.A./R.I.A. flat bottom plates, Costar, Cambridge, MA, USA). 139H2 (IgG) and SPan-1 (IgM) antibodies were used to identify MUC1 and sialyl-Lewis^a, respectively. In direct binding assays, antigen was adsorbed directly to the wells. Both 19-9 (IgG) and SPan-1 gave similar results in identifying sialyl-Lewis^a. Horseradish peroxidase conjugated second antibody against IgM (Sigma Chem. Co.), or against IgG (Zymed Lab., S. San Francisco, CA, USA) was used to quantitate binding.

2.5. Immunoprecipitation and Western blotting

Immunoprecipitation was performed as previously described [3,13]. To standardise the experiment, equal protein concentrations, for all samples to be compared, were used prior to the immunoprecipitation. SPan-1 or myeloma (SP-2/0-Ag 14) ascites (1/100 dilution) were used. SDS-PAGE and Western blotting with ^{125}I-protein A were performed as previously described [13] except that a Mighty Small II Vertical Slab Unit (Pharmacia Biotech, Inc., Piscataway, NJ, USA) and PVDF membranes (Trans-Blot Transfer Medium, Bio-Rad, Hercules, CA, USA) were used. Membranes were probed with MUC1 directed 139H2 antibody (1/500 dilution of ascites).

2.6. RNA isolation and slot blots

RNA was isolated with a RNeasy total RNA kit (Qiagen, Chatsworth, CA, USA). Slot blotting was performed as previously described [14]. The probe for MUC1 (PUM 24P) was a gift from D. Swallow (MRC Human Biochemical Genetics Unit, University College London, London, UK) and the probe for GAPDH was developed in our laboratory. Probes were radiolabelled by the random primer reaction using a kit

(RadPrime DNA Labeling Systems, Gaithersberg, MD, USA).

2.7. Analysis of autoradiograms

Radiograms of RNA slot blots were scanned with a UMAX UC1260 scanner and MagicScan DA1.1.1 software. Densities of bands were quantitated with NIH Image 1.54 software.

2.8. Binding of cells to E-selectin coated microtitre plates

Binding of SW1990 cells to E-selectin was examined as previously described [6]. Single cell suspensions were incubated with recombinant E-selectin–IgG or control CD7-IgG (gifts from A. Aruffo, Bristol-Myers Squibb, Evansville, IA, USA) in coated wells of 96-well microtitre plates. Attached cells were quantitated with MTT [7]. In some experiments, wells were pre-incubated with 10 μg/ml anti-E-selectin antibody. In other experiments, cells were pre-incubated with CSLEX-1 (1/10 dilution of pure antibody solution) or SPan-1 or SNH3 ascites (1/50 in 0.02 mg/ml bovine serum albumin (BSA) in serum-free media) for 30 min at 37°C.

3. Results

3.1. Release of MUC1-associated sialyl-Lewis^a and PKA and PKC activities

Forskolin increased the activity of PKA, an enzyme that is activated by cyclic AMP, within the first 4 h of forskolin treatment (Fig. 1a). PKA activity in the cell homogenates reached a plateau at approximately +24 h. The PKA inhibitor peptide TYADFIASGRTGRRNAI prevented the forskolin-induced increase in PKA activity (data not shown). The greatest increase in release of MUC1-associated sialyl-Lewis^a from whole cells (+42–48 h) occurred after PKA activity had reached a plateau (+24 h). An inhibitor of PKA, H8 [15], blocked this stimulated release. The activity of PKC doubled within the first hour after addition of PMA (Fig. 1). Unlike

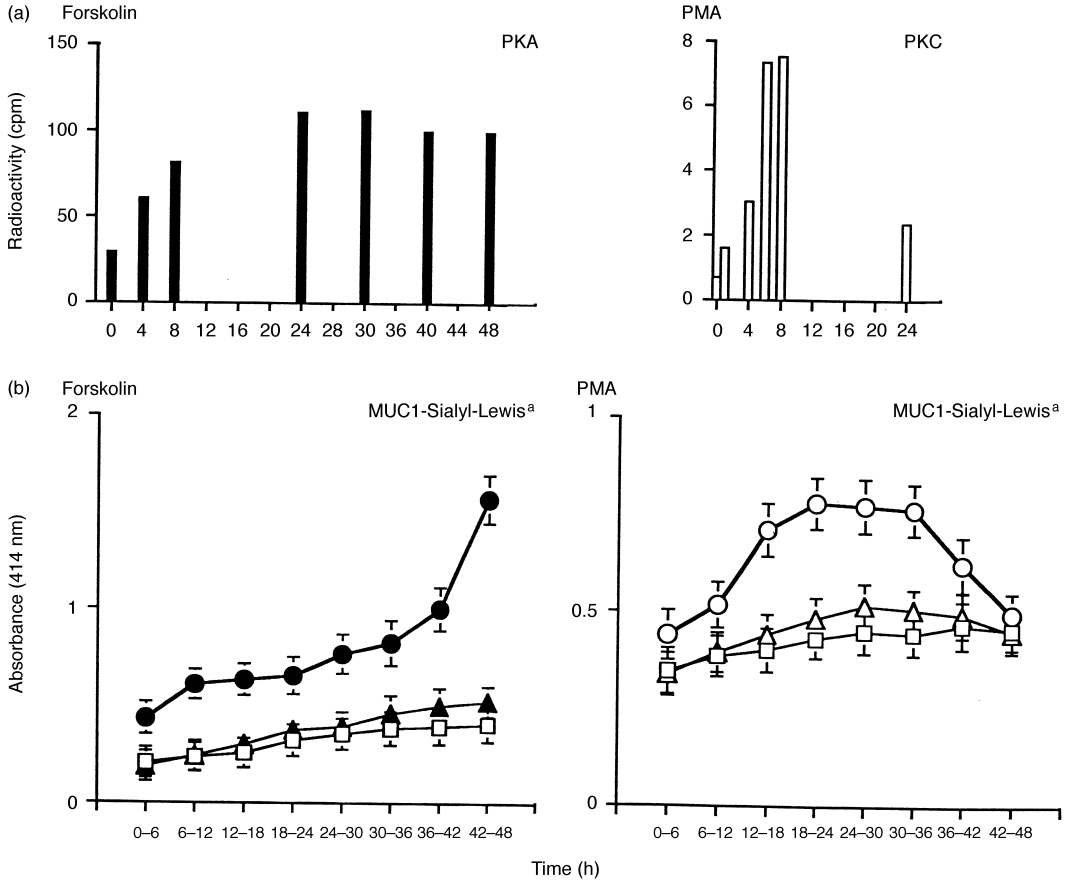

Fig. 1. Time course of PKA and PKC activities and release of antigen. (a) PKA (solid bars) and PKC (open bars) activities of homogenates were determined with the substrate peptides LRRASLG (Kemptide) and QKRPSQRSKYL, respectively after treatment with forskolin (10 μg/ml) or PMA (100 ng/ml). (b) Conditioned media of cells treated with forskolin (10 μg/ml, ●) or PMA (100 ng/ml, ○) were collected every 6 h, centrifuged and concentrated for immunoassays. Double determinant immunoassays were performed to quantitate the levels of MUC1-associated sialyl-Lewis^a. The capture and reporter antibodies were 139H2 (MUC1) and SPan-1 (sialyl-Lewis^a), respectively. The inhibitors H7 (50 μM, △) and H8 (120 μM, ▲) were used. Control cells (□). Each point is the mean ± standard deviation (S.D.) ($n=3$).

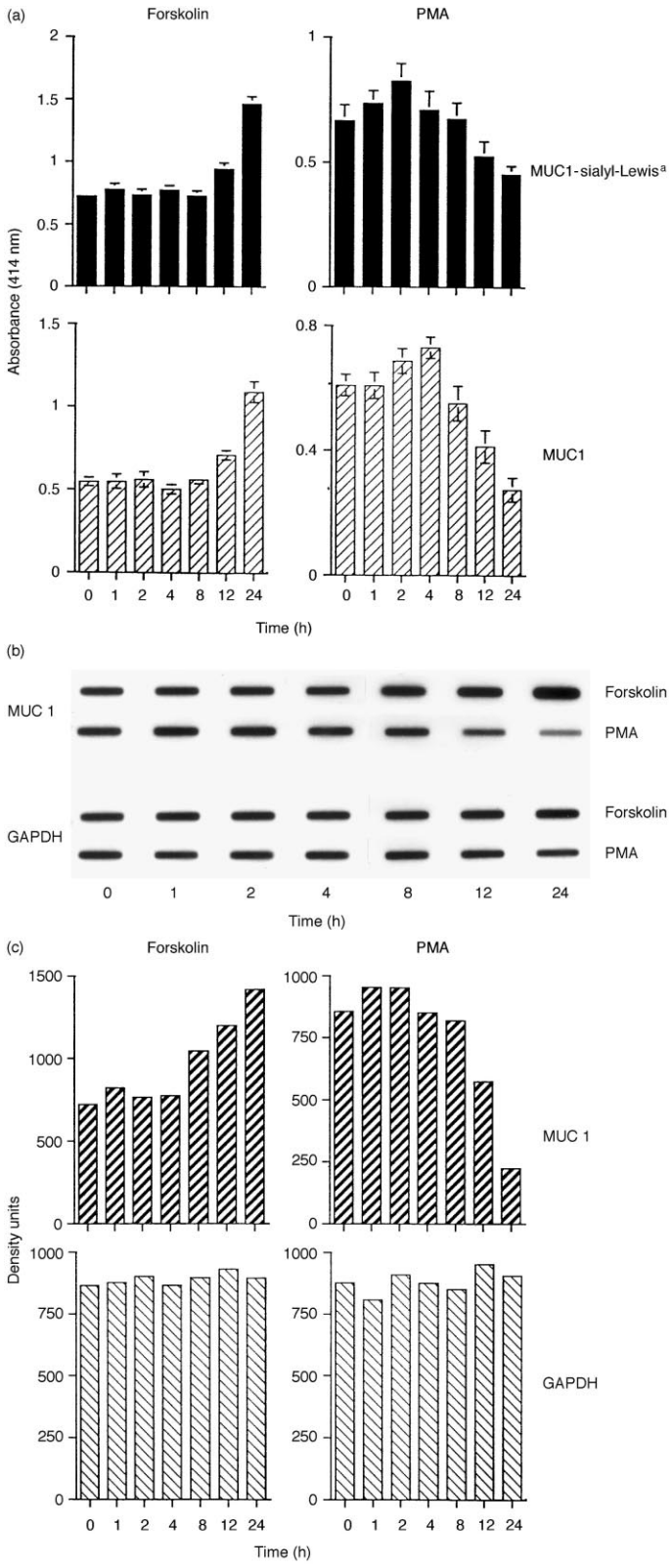

Fig. 2. Antigen and RNA levels. (a) Cytosol levels of antigen. Cells were treated with forskolin (10 μg/ml) or PMA (100 ng/ml). Levels of MUC1-associated sialyl-Lewis[a] antigen (■) were determined with a double determinant immunoassay with 139H2 as the capture antibody and SPan-1 as the reporter antibody. Levels of MUC1 antigen (▨) were determined with a direct binding immunoassay using 139H2. Each bar is the mean ± standard deviation (S.D.) ($n=3$). (b) Autoradiograms of slot blots of RNA. RNA from forskolin and PMA-treated cells were hybridised with MUC1 and GAPDH probes. (c) Densities of radiograms of RNA slot blots.

PKA activity, which remained high for at least 48 h, PKC activity was below its maximum level at (approximately 30%) +24 h. The PKC inhibitor peptide RFARKGALRQKNV blocked the increase in PKC activity produced by PMA (data not shown). Release of MUC1-associated sialyl-Lewis[a] from PMA treated cells dropped sharply at +36 h reaching control values at +48 h (Fig. 1b). H7, an inhibitor of PKC [15], blocked PMA-stimulated release.

3.2. Cellular levels of antigens and MUC1 mRNA

Forskolin also increased the cellular content of antigen. A significant increase in cytosol levels of MUC1-associated sialyl-Lewis[a] in forskolin treated cells was not seen before +12 h (Fig. 2a) with the greatest change occurring between +12 and +24 h. In PMA-treated cells, there was a slight, but reproducible, increase in the two antigens between +2–4 h. However, the most notable change in cytosol levels was a reduction by +24 h to less than control values. MUC1 mRNA levels in forskolin treated cells were higher at the +8 h time point than at +4 h and continued to increase (Fig. 2b and c). PMA produced a slight, but reproducible, increase in MUC1 mRNA at approximately +1–+4 h (+1 and +2 h in Fig. 2b and c). However, MUC1 mRNA levels of PMA-treated cells were markedly lower than control levels at +12 and +24 h.

3.3. Molecular characteristics of released and cytoplasmic antigens

Since sialyl-Lewis[a] could be present on both glycolipids [16] and glycoproteins [1] we wanted to confirm that the changes in sialyl-Lewis[a] produced by forskolin and PMA were in mucin-associated antigen. Western blots of SPan-1 immunoprecipitated antigens probed with the MUC1-directed antibody 139H2 showed that antigen from media was of high molecular weight, remaining in the 4% stacking gel or at the top of the 7% resolving gel, thus resembling mucins (Fig. 3). Antigen immunoprecipitated from whole cells also showed time-dependent changes in levels that were similar to those in the cytosol. MUC1 antigen from whole cells differed from that found in media in having two additional faint lower molecular weight bands [14].

3.4. Effects of Act D and protein kinase inhibitors on antigen and MUC1 mRNA

Act D blocked the increase in MUC1 mRNA that was produced by forskolin (Fig. 4b and c). Act D also blocked the increase in MUC1-associated sialyl-Lewis[a] and MUC1 antigens (Fig. 4a). The forskolin induction of sialyl-Lewis[a] and MUC1 antigens and MUC1 mRNA were abolished by H8. In contrast, H7 partially

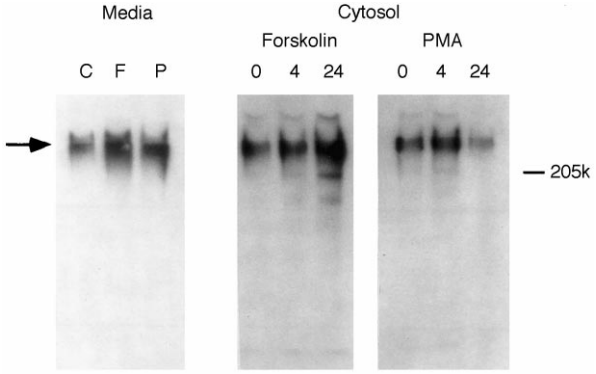

Fig. 3. Western blots of immunoprecipitation of sialyl-Lewis[a] antigen from media and cell lysates. Conditioned media were collected from control cells (C) or cells treated for 24 h with forskolin (10 μg/ml forskolin, F) or PMA (100 ng/ml, P). Control cells (0) and cells treated 4 or 24 h with forskolin and PMA were lysed in immunoprecipitation media. Sialyl-Lewis[a] antigen was immunoprecipitated with SPan-1 antibody, separated by SDS-PAGE then transferred to PVDF membranes. Membranes were probed with MUC1-directed 139H2 antibody. The 205 kDa marker was myosin. The arrow indicates the interface between the 4% stacking and 7% resolving gels.

blocked the effect of PMA in reducing the levels of the two antigens and MUC1 RNA. The three inhibitors had no detectable effect on GAPDH RNA.

3.5. Effects of PMA on cell surface antigen and cell binding to E-selectin

We wanted to determine if reducing the cell content of sialyl-Lewis[a] in SW1990 cells would affect cell binding to the endothelial cell adhesion molecule, E-selectin. PMA reduced surface expression of both sialyl-Lewis[a] and MUC1 antigens below control levels after 48 h (Fig. 5a). The cells also had a reduced ability to bind to E-selectin (Fig. 5). This correlation of binding with surface sialyl-Lewis[a] was consistent with earlier results [6] where surface sialyl-Lewis[a] levels were reduced by an inhibitor of O-glycosylation. The importance of sialyl-Lewis[a] for selectin binding was confirmed in experiments where cells that had been pretreated with an antibody against sialyl-Lewis[a] showed greatly reduced binding to E-selectin (Fig. 5b, SPan-1). Moreover SNH3 antibody (sialyl-Lewis[x], IgM, 10) did not affect binding to E-selectin (Fig. 5b). Similar results were obtained with CSLEX1 (sialyl-Lewis[x], IgM, 3; data not shown). Both antibodies react with SW1990 cells [3,6]. Pre-incubation of the adsorbed E-selectin with an antibody directed against E-selectin prevented cell binding.

4. Discussion

Our results suggest that activation of either the cyclic AMP/PKA or the diacylglycerol/PKC will elevate the

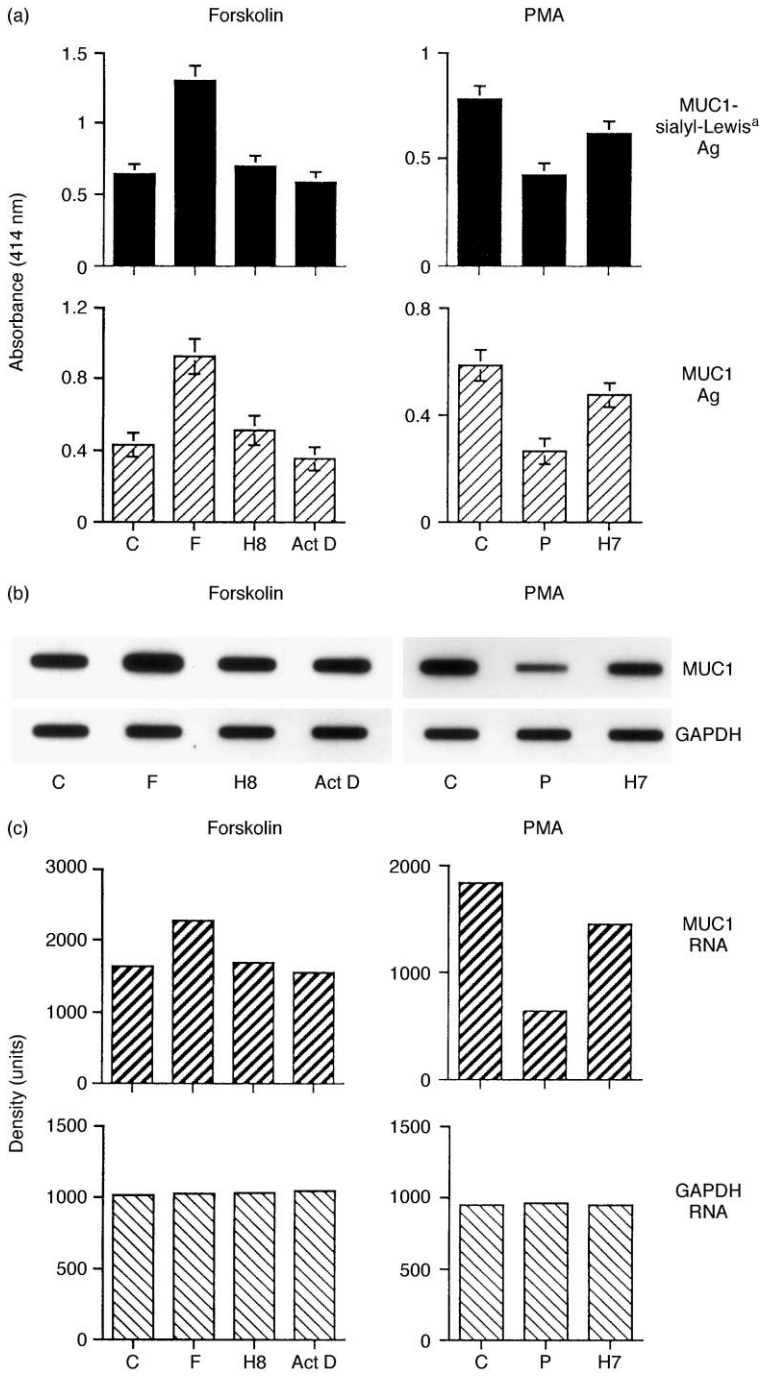

Fig. 4. Effects of H7, H8, and Act D on antigen and MUC1 RNA levels. (a) Antigen levels were determined by either double determinant or direct binding immunoassays. Each antigen data bar is the mean ± standard deviation (S.D.) ($n = 3$). Total RNA was isolated from control cells (C) and cells treated for 24 h with forskolin (10 μg/ml, F) or PMA (100 ng/ml, P) with or without inhibitor. The inhibitors were Act D (10 μM), H7 (50 μM) and H8 (120 μM). (b) Autoradigrams of slot blots. (c) Densities of slots of radiograms shown in (b).

release of MUC1-associated sialyl-Lewis[a] from SW1990 pancreatic cancer cells. However, the two pathways produced opposite effects on the cellular content of antigen and MUC1 RNA. During the time period of increased release there was an increase in the level of MUC1 RNA in forskolin-treated cells. It is possible that in forskolin-stimulated cells, release is a function of both increased cellular levels and direct stimulation of a specific release mechanism. In contrast, although PMA produced a small, but reproducible, increase in the cell content of antigen and in MUC1 RNA (+2 to +4 h) the most significant change was a fall in levels, which became measurable at +8 h. After 24 h cellular MUC1 RNA levels were approximately 25% of control levels.

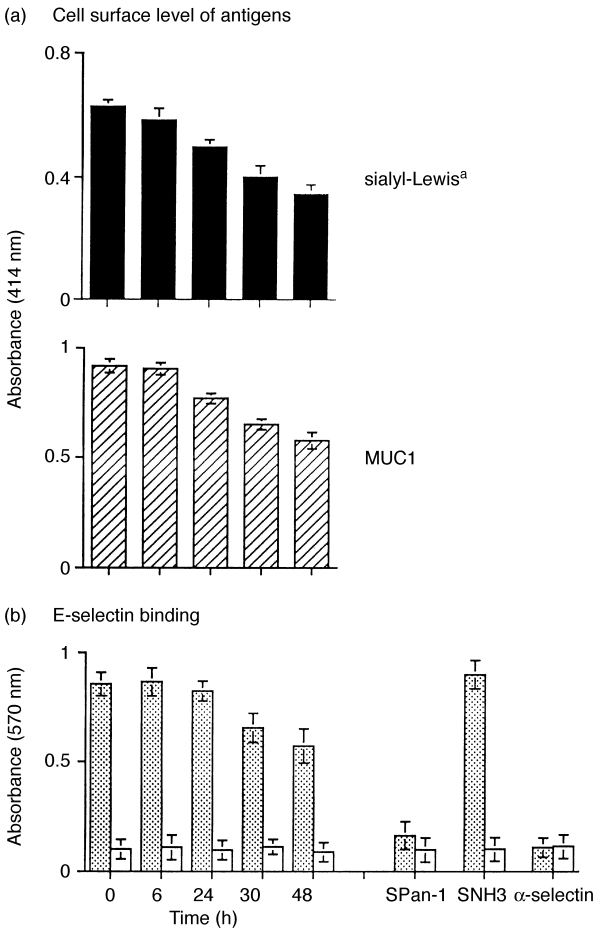

Fig. 5. Effects of PMA on cell surface levels of antigen and E-selectin binding. (a) Sialyl-Lewis[a] and MUC1 antigens on the surfaces of cells were determined by immunoassays of cells as previously described. 19-9 (IgG) and 139H2 (IgG) monoclonal antibodies were used to quantitate sialyl-Lewis[a] and MUC1 antigens, respectively. Each bar is the mean ± standard deviation (S.D.) ($n=3$). (b) Binding of treated cells to E-selectin was performed by incubating dissociated cells with E-selectin–IgG (shaded bars) or CD7–IgG (open bars) adsorbed to microtitre plates. Involvement of sialyl-Lewis antigens in the binding was determined by pre-incubating untreated cells with another sialyl-Lewis[a] directed antibody, SPan-1 (IgM), or with sialyl-Lewis[x] directed SNH3 (IgM). Each bar is the mean ± standard deviation (S.D.) ($n=6$).

This fall in cellular content may be the main factor contributing to the fall in release that was seen in PMA-treated cells.

MUC1 is a membrane-associated mucin that contains both transmembrane and cytoplasmic domains. The MUC1 mucin that is released from the cell does not contain the cytoplasmic domain [17]. Despite the importance of this molecule as the basis of several serological diagnostic and prognostic tests for breast and pancreatic cancer (e.g. CA15-3, CA19-9, and SPan-1; [1,2,18]), the precise mechanism of its release from cells and the factors that alter the levels of release are still not known. It is thought that the process may involve proteolysis [17]. In Chinese hamster ovary cells, PMA induces shedding of a number of membrane proteins by a mechanism involving metalloproteases [19]. SW1990 cells would be a good model system to compare the mechanism(s) of forskolin- and PMA-stimulated release of MUC1.

Changes in the expression of the carbohydrate Lewis antigens involve the action of glycosyltransferases, with or without, changes in the levels of the protein backbone (MUC1 in our study). In the case of a cell density-induced increase in the sialyl-Lewis[a] level, we were not able to measure a similar increase in the level of MUC1 protein [14]. Little is known about the mechanisms that produce short-term changes in the synthesis of sialylated-Lewis antigens. Recently, PMA was shown to increase the synthesis of sialyl-Lewis[x] in T cells, as well as to increase the steady-state levels of mRNA for the FucT-VII fucosyltransferase [20]. Our present results show that the sialyl-Lewis[a] antigen changes in response to either forskolin or PMA in the same direction and with roughly the same time course as MUC1 antigen and mRNA. Moreover, inhibitors affected the levels of the carbohydrate, protein and mRNA in similar ways. However, we cannot exclude the possibility that there is some dissociation between the changes in the carbohydrate versus protein portions of the molecule with either forskolin or PMA or both. Additional studies are in progress to determine if sialyl-Lewis[a] is on other types of mucins, in addition to MUC1.

We have recently shown that forskolin can also increase cellular levels of sialyl-Lewis[a] levels *in vivo* [21]. When athymic mice with SW1990 xenografts were treated with forskolin, the CA19-9 levels in both the circulation and xenograft tissues were greatly elevated. We have also shown that the level of a tumour-specific antigen in pancreas, Nd2, can also be elevated by forskolin [22]. Nd2 antibody has a high sensitivity in radioimaging pancreatic exocrine tumours in clinical trials [23] and in producing tumour regression in animal models when conjugated to doxorubicin [24]. Thus, activation of the cyclic AMP pathway could improve the efficacy of tests and therapeutic modalities based on circulating and cell-associated tumour antigens. The decrease in surface expression of mucin-associated sialyl-Lewis[a] that was produced by PMA also has clinical potential. Because sialyl-Lewis[a] plays an important role in pancreatic tumour cell extravasation [4–6], down regulation by activation of the diacylglycerol pathway could be a means of reducing tumour cell metastasis.

Acknowledgements

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